

The single CH domain of calponin is neither sufficient nor necessary for F-actin binding

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SUMMARY

Calponins have been implicated in the regulation of actomyosin interactions in smooth muscle cells, cytoskeletal organisation in nonmuscle cells, and the control of neurite outgrowth. Domains homologous to the amino-terminal region of calponin have been identified in a variety of actin cross-linking proteins and signal transduction molecules, and by inference these 'calponin homology (CH) domains' have been assumed to participate in actin binding.

We here report on the actin binding activities of the subdomains of the calponin molecule. All three mammalian isoforms of calponin (basic *h1*, neutral *h2* and acidic) possess a single CH domain at their amino terminus as well as three tandem repeats proximal to the carboxyl terminus. Calponin *h2* differs, however, from *h1* in lacking a consensus actin-binding motif in the region 142-163, between the CH domain and the tandem repeats, which in *h1* calponin can be chemically cross-linked to actin. Despite the absence of this consensus actin-binding motif, recombinant full-length *h2* calponin co-sediments in vitro with F-actin, suggesting the presence of another binding

site in the molecule. It could be shown that this binding site resides in the C-terminal tandem repeats and not in the CH domain. Thus, constructs of *h2* calponin bearing partial or complete deletions of the triple repeated sequences failed to co-localise with actin stress fibres despite the presence of a CH domain. Deletion of the acidic carboxyl terminus, beyond the repeats, increased actin binding, suggesting that the carboxy-terminal tail may modulate actin association. Results obtained from transient transfections of amino- and carboxy-terminal truncations in *h1* calponin were consistent with the established location of the actin binding motif outside and carboxy-terminal to the CH domain, and confirm that the presence of a single CH domain alone is neither sufficient nor necessary to mediate actin binding. Instead, the carboxy-terminal tandem repeats of *h1* and *h2* calponin are shown to harbour a second, independent actin binding motif.

Key words: Calponin, CH domain, Localisation, Transfection, Basic calponin isoform (*h1*), Neutral calponin isoform (*h2*)

INTRODUCTION

The calponin (CaP) family of F-actin-, tropomyosin- and S100-binding proteins contains three genetic variants, a smooth muscle-specific variant termed *h1* or basic CaP (Takahashi et al., 1988), a neutral variant of similar molecular mass termed *h2* (Strasser et al., 1993) and an acidic variant (Applegate et al., 1994), which is not tissue-specific and that is particularly enriched in brain (Trabelsi-Terzidis et al., 1995). The three variants share high sequence identity (>70%) within the first 273 amino acid residues, but differ in their highly acidic carboxy-terminal tail sequences (Takahashi and Nadal-Ginard, 1991; Strasser et al., 1993; Applegate et al., 1994; Maguchi et al., 1995; reviewed in Gimona and Small, 1996). *h1* CaP has also been shown to interact in vitro with myosin (Szymanski and Tao, 1993, 1997), caldesmon (Vancompernelle et al., 1990; Graceffa et al., 1996), desmin (Mabuchi et al., 1997), tubulin (Fujii et al., 1997) and phospholipids (Bogatcheva et al., 1995; Fujii et al., 1995).

Partial sequence similarities exist between calponin and the SM 22 family of smooth muscle proteins (Lees-Miller et al., 1987; Pearlstone et al., 1987), UNC-87, the bodywall muscle protein of *C. elegans*, (Goetinck and Waterston, 1994a,b) mp 20, a protein from *Drosophila* flight muscle, np 25, a protein of unknown function identified in rat brain and an immunogenic protein of 45 kDa found in the parasite *Onchocerca volvulus* (see Gimona and Small, 1996 and references therein) as well as with a large number of CaP- and SM 22-related proteins (T. Stradal and M. Gimona, unpublished). A region spanning residues 32 to 127 in CaP is also present in a variety of molecules involved in signal transduction (Vav, IQGAP 1 and 2, Ras GAP-like protein, human ORFP) and has been termed the 'calponin homology (CH) domain' (Castresana and Saraste, 1995). CH domains share about 30% sequence similarity with the actin-binding domains (ABD) present in α -actinin, filamin, fimbrin and spectrin (Castresana and Saraste, 1995). Although the presence of this domain has been used to ascribe an F-actin-binding

function to the latter GAPs and GEFs (Hart et al., 1996; Brill et al., 1996), this region does not correspond to or overlap with the known actin binding domain of *h1* CaP, which embraces residues 142-163 (Mezgueldi et al., 1992, 1995). Moreover, established actin-binding proteins that use the region analogous to the CH domain for binding to F-actin contain this domain in a tandem repeat and form parallel or anti-parallel dimers (Vandekerckhove, 1990; Matsudaira, 1991; Pollard et al., 1994), which thus contain multiple CH domains. Notably, IQGAP 1 has recently been demonstrated to exist as a dimer which binds to actin (Bashour et al., 1997) and in the proto-oncogene *Vav* the leucine-rich helix-loop-helix motif, contained within the single amino-terminal CH domain, has been implicated in dimer formation (see Romero and Fischer, 1996 for a review). Significantly, the loss of the amino-terminal region in *Vav* containing the CH domain activates the transforming potential of the molecule (Katzav et al., 1991).

Whereas *h1* CaP is believed to influence the contraction/relaxation cycle in smooth muscle by blocking the S1 binding site for myosin on actin (EL-Mezgueldi and Marston, 1996), little experimental data is available for *h2* CaP. However, from the limited localisation data available, this CaP variant has been implicated in the organisation of the actin cytoskeleton (Fukui et al., 1997) and it has been suggested that acidic CaP may play a role in controlling neurite outgrowth and govern neuronal regeneration (Ferhat et al., 1996).

In order to gain more insight into the functional significance of the different domains of CaP, and in particular the involvement of the calponin CH domain in actin binding, we have transfected epitope-tagged constructs of *h1* and *h2* CaP into fibroblasts and assayed for their association with F-actin in cultured fibroblasts. We show that the single CH domain present in CaP and the sequence-related protein SM 22 does not confer binding to F-actin in co-sedimentation assays, or association with the actin cytoskeleton in cultured cells. Biochemical data using recombinant fragments of *h1* and *h2* CaP and SM 22 support the cell biological findings and in addition pinpoint the proximal carboxy-terminal tandem-repeat region as a new actin-targeting domain.

MATERIALS AND METHODS

Construction of plasmids

Standard protocols were used for all cDNA manipulations (Sambrook et al., 1989). PCR reactions for each clone, using Taq DNA polymerase, were performed using specific 5'-primers containing an *Xba*I site and corresponding 3'-primers containing a *Bam*HI site, using mouse uterus *h1* or *h2* CaP DNA or mouse uterus SM 22 DNA cloned into the vector pMW 172 as a template (Strasser et al., 1993). All calponin constructs were cloned in-frame *Xba*I to *Bam*HI into the pCGN expression vector containing the HA-tag sequence at the 5'-end as described (Gimona et al., 1995). All tagged constructs therefore represent amino-terminal fusion proteins. A stop codon was introduced at the 3'-end of each clone and all constructs were sequenced from both strands using a LI-Cor model 4000 automated sequencer (MWG Biotech, Germany).

Proteins

Rabbit skeletal muscle actin and turkey gizzard smooth muscle actin were prepared from acetone powder according to Spudich and Watt (1971) and Strzelecka-Golaszewska et al. (1975), respectively.

Recombinant *h2* CaP (1-305) and three carboxy-terminal mutations (1-262, 1-186, 1-161) were expressed as non-fusions at high levels in *E. coli* and purified in a single chromatography step using an SP-HiTrap cation exchange column (Pharmacia, Sweden). Lysates were generated by disrupting the bacterial cells in a French press as described earlier (Strasser et al., 1993), resulting in more than 85% of the expressed proteins in the soluble fraction. Soluble proteins after centrifugation at 15000 *g* were applied directly onto the SP column and eluted with a linear 20 mM to 400 mM NaCl gradient. Purified proteins were then dialysed into the appropriate assay buffers for co-sedimentation.

Actin binding assays

Co-sedimentation assays with smooth or skeletal muscle F-actin were performed in a buffer containing 20 mM imidazole, 50 mM NaCl, 2 mM MgCl₂, pH 7.0. Proteins at a molar ratio of 1:3 (recombinant protein:F-actin) were incubated at 25°C for 30 minutes and pelleted at 100000 *g* for 30 minutes using an air-driven ultracentrifuge (Beckman Instruments). Pellets were resuspended in the same buffer in the starting volume.

Cell culture, transfection and immunofluorescence microscopy

NIH 3T3 and REF 52 fibroblasts were grown in high glucose (4500 mg/l) DMEM supplemented with 10% FBS (Hyclone, Utah, USA), penicillin/streptomycin (Gibco, Austria) at 37°C and 5% CO₂. Cells at 70% confluence were transfected using the Lipofectamine (Life Technologies, Austria) method in the absence (NIH 3T3) or presence (REF 52) of serum, essentially as described (Gimona et al., 1995). For transient expression, cells grown and transfected on 12 mm coverslips were screened 24 hours post-transfection by immunofluorescence. Cells co-transfected with the plasmid pCDNA for the selection of stable cell lines were passaged after similar transfection times and cells resistant to 1 mg/ml G-418 and expressing the transfected transgene were identified by western blotting. For immunofluorescence microscopy cells were cultured on 12 mm glass coverslips. Cells were washed three times in PBS (138 mM NaCl, 26 mM KCl, 84 mM Na₂HPO₄, 14 mM KH₂PO₄, pH 7.4), extracted in 3.7% PFA/0.3% Triton X-100 in PBS for 5 minutes, and fixed in 3.7% PFA (Merck, Germany) in PBS for 30 minutes. Tagged CaP fragments were visualised using the anti-tag antibody and GaM-rhodamine secondary antibody (Jackson) in PBS. Monoclonal anti-HA (clone 12CA5) was described (Gimona et al., 1995) and produced as mouse ascites fluid at Cold Spring Harbor Laboratory (NY). Cy3-labelled secondary fluorescent antibodies were from Jackson Laboratories (Dianova, Germany) and FITC-phalloidin was a gift from Dr Faulstich (Munich). Fluorescent images were recorded on a Zeiss Axiophot microscope using a 63× oil immersion lens and Kodak P3200 or P400 Tmax film.

Electrophoresis and western blotting:

Analytical SDS-gel electrophoresis on 8% to 22% gradient polyacrylamide mini-slab gels and western blotting onto nitrocellulose (Amersham) was performed as described elsewhere (Gimona et al., 1990). Transferred proteins were visualised using horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence detection system (Amersham).

RESULTS

Domains of calponin

For the purpose of the present study we have divided CaP into four subdomains based on the data available for the *h1* variant (Fig. 1): (1) an amino-terminal CH domain (CH); (2) an actin-binding site (ABS); (3) three 29-residue tandem repeats; and (4)

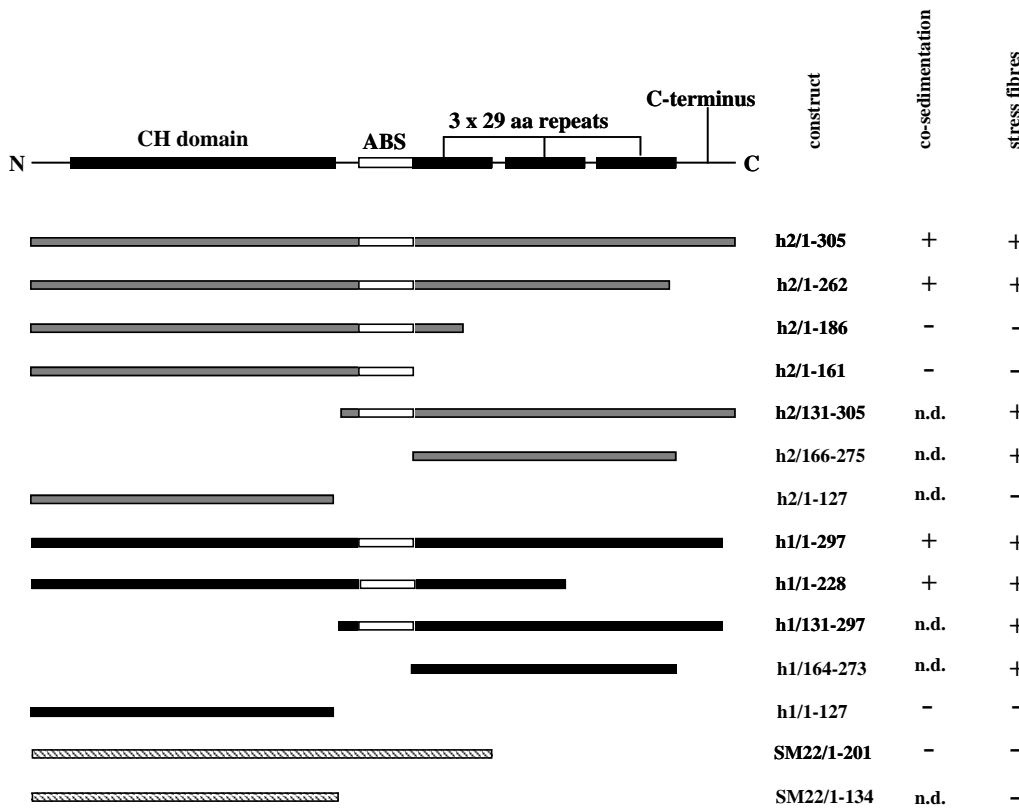


Fig. 1. Domain structure of mammalian calponin. Schematic representation of the delineated functional and structural domains in *h1* calponin (top) and the corresponding constructs of *h1* and *h2* CaP, and SM 22 used in this study. Primary domains are the CH domain (CH), the actin binding site (ABS), the adjacent triple repeats and the carboxy-terminal tail. The activities (+ or -) of the constructs in co-sedimentation and stress fibre association are summarised on the right. n.d., not determined.

a carboxy terminal tail. All three CaP variants display a high level of sequence identity along most of the length of the molecule, with the exception of the very carboxy-terminal residues, beyond the three repeats. In the region of *h2* CaP corresponding to the actin binding motif in *h1* and acidic CaP (Fig. 2) there is, however, a cluster of several non-conservative amino acid exchanges. Notably, three exchanges (A145S, R151N and K156T) are unique for the *h2* variant. Mutation of Ala145 has been shown to strongly reduce the inhibitory activity of *h1* CaP on the actin-activated ATPase of myosin (EL-Mezgueldi et al., 1996) and Lys156 was identified as the residue most crucial for actin binding in chicken α -CaP (Gong et al., 1993).

Truncated constructs of *h1* and *h2* CaP were generated as indicated in Fig. 1, and among these constructs all carboxy-

human <i>h1</i>	V	K	Y	A	E	K	Q	E	R	K	F	E	P	G	K	L	R	E	G	R	N	I
mouse <i>h1</i>	V	K	Y	A	E	K	Q	E	R	R	F	E	P	E	K	L	R	E	G	R	N	I
human acidic	V	K	Y	A	E	K	Q	T	R	R	F	D	E	G	K	L	K	A	G	Q	S	V
rat acidic	V	K	Y	A	E	K	Q	T	R	R	F	D	E	G	K	L	K	A	G	Q	S	V
human <i>h2</i>	V	K	Y	S	E	K	Q	E	R	N	F	D	D	A	T	M	K	A	G	Q	C	G
mouse <i>h2</i>	V	K	Y	S	E	K	Q	E	R	N	F	D	D	A	T	M	K	A	G	Q	C	V
				↑					↑					↑								
				145					151					156								

Fig. 2. The actin binding site in calponins. Comparison of the amino acid sequences of CaPs in the region corresponding to the delineated actin binding site (ABS) of *h1* CaP, spanning residues 142-163. Note that all exchanges but the A145S substitution affect charged residues. Amino acid substitutions occurring only in *h2* CaP are highlighted by gray boxes. Sequence comparison adapted from Gimona and Small (1996).

terminal truncations of *h2* CaP as well as full-length SM 22 were additionally produced as recombinant, non-fusion proteins in *E. coli* for assays of actin binding in vitro.

Actin binding

Co-sedimentation assays using either skeletal or smooth muscle actin demonstrated that, like *h1* CaP, full-length

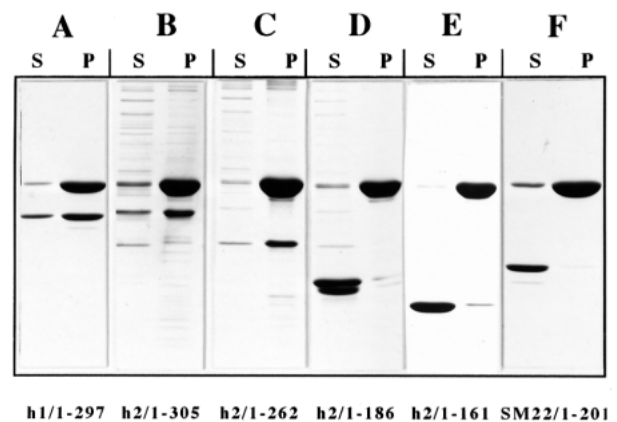


Fig. 3. Co-sedimentation assay. Coomassie blue-stained 8%-22% polyacrylamide gel showing soluble (S) and pelleted (P) material following high speed centrifugation at 100000 g. Smooth muscle actin was incubated with either native porcine calponin (A) or recombinant *h2*/1-305 (B), *h2*/1-262 (C), *h2*/1-186 (D), *h2*/1-161 (E) or SM 22 (F). *h1* and *h2* CaP co-sediment with smooth muscle F-actin, as does the carboxy-terminal truncation *h2*/1-262. By contrast, recombinant SM 22 and the two *h2* constructs 1-186 and 1-161 remain in the supernatant.

recombinant *h2* CaP (*h2/1-305*) and the fragment *h2/1-262* lacking the acidic carboxyl terminus pelleted together with F-actin after centrifugation at 100000 *g* for 30 minutes (Fig. 3A-C). However, fragments *h2/1-186* and *h2/1-161* bearing further truncations at the carboxy-terminal end failed to co-sediment with F-actin under the same conditions (Fig. 3D,E). Similarly, full-length recombinant SM 22 (1-201) did not co-sediment with either smooth (Fig. 3F) or skeletal muscle actin (not shown). The addition of smooth muscle tropomyosin to the assay had no influence on the binding of either recombinant *h1* CaP, *h2* CaP or SM 22 (M. Gimona and P. Strasser, unpublished). Increasing the ionic strength from 50 to 100 mM NaCl did not alter the co-sedimentation behaviour of any of the constructs.

Expression and subcellular localization of *h1* and *h2* calponin

Western blot analysis using the anti-HA tag antibody demonstrated that all constructs were expressed at low to intermediate levels (Fig. 4) in transfected NIH 3T3 or REF 52 cell lines. The migration of the expressed proteins matched the estimated molecular masses of the tagged constructs, taken as 2 kDa heavier than the recombinant controls. Expression was further analysed by western blotting of two-dimensional gels containing whole-cell extracts of the various stable cell lines. The anti-tag antibody specifically recognised a single spot at the expected molecular size range and corresponding pI value. In contrast to native smooth muscle CaP, we did not observe multiple spots on 2D-gels for any of the transiently or stably expressing cell lines (Gimona et al., 1992; M. Gimona, unpublished observations).

Full-length *h1* CaP (*h1/1-297*) was observed to decorate the actin stress fibres of transiently transfected REF 52 cells (Fig. 5A) as did the shorter constructs *h1/1-228* (Fig. 5B) and *h1/131-297* (Fig. 5C), demonstrating that truncation of either side of the actin-binding motif, involving in one case the complete deletion of the CH domain, does not affect actin association.

As for *h1* CaP, full-length acidic CaP (Fig. 6A) and *h2* CaP (Fig. 6C) associated with the actin stress fibres of transiently transfected REF 52 fibroblasts, but were absent from the F-actin-containing lamellipodia at the cell periphery (see Fig. 6C,D). In addition, *h2* CaP appeared to be more concentrated at the ends of stress fibers. Deletion of the acidic carboxyl

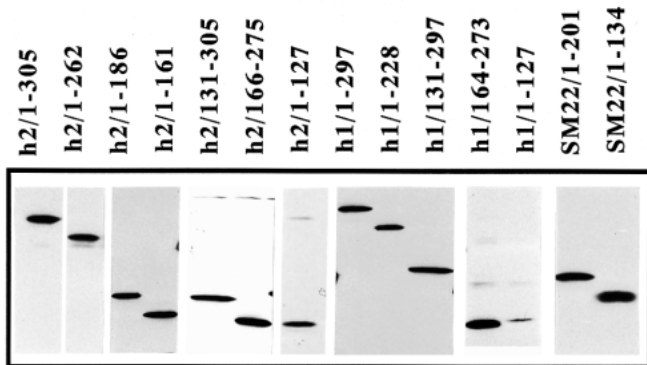


Fig. 4. Forced expression of CaP constructs. Western blot of whole cell lysates from transiently transfected REF 52 cells probed with the HA-epitope tag-specific antibody 12CA5.

terminus from *h2* CaP enhanced the actin association and the corresponding construct (*h2/1-262*) decorated the entire length of the stress fibres more homogeneously than the full-length molecule (Fig. 6E). Further truncation of the *h2* CaP molecule from the carboxyl terminal end (*h2/1-186* and *h2/1-161*) almost completely abolished the ability of the respective constructs to associate with F-actin in transfected NIH 3T3 fibroblasts (Fig. 7A,C), although both constructs contained a complete CH domain. Immunoreactive material was also accumulated around and inside the nucleus.

SM 22 was also diffusely localised in the cytoplasm (Fig. 7E,F) with an additional strong fluorescent signal in the nuclear region. No stress fibre association of SM 22 was obtained in transiently transfected REF 52 cells nor in any of the 16

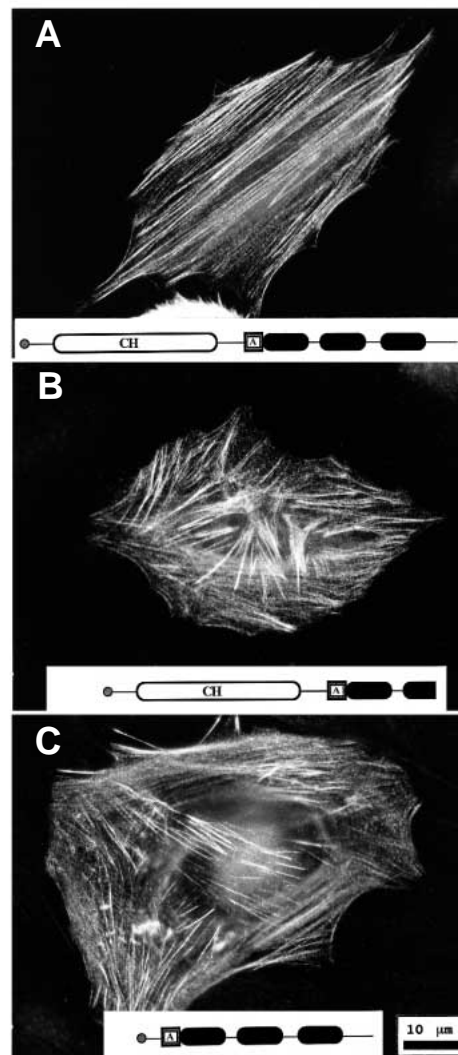


Fig. 5. *H1* CaP binds to stress fibres of fibroblasts both in the presence and the absence of a CH domain. Association of the three *h1* constructs *h1/1-297* (A), *1-228* (B) and *131-297* (C), transiently transfected into REF 52 cells along F-actin stress fibres. Schematic insets indicate calponin domains (see also Fig. 1). Small dots at the amino-terminal ends of the insets symbolise the location of the epitope tag sequence. Note the stress fibre association in the *h1* construct lacking the entire CH domain (C). All anti-tag antibody/GaM Cy2.

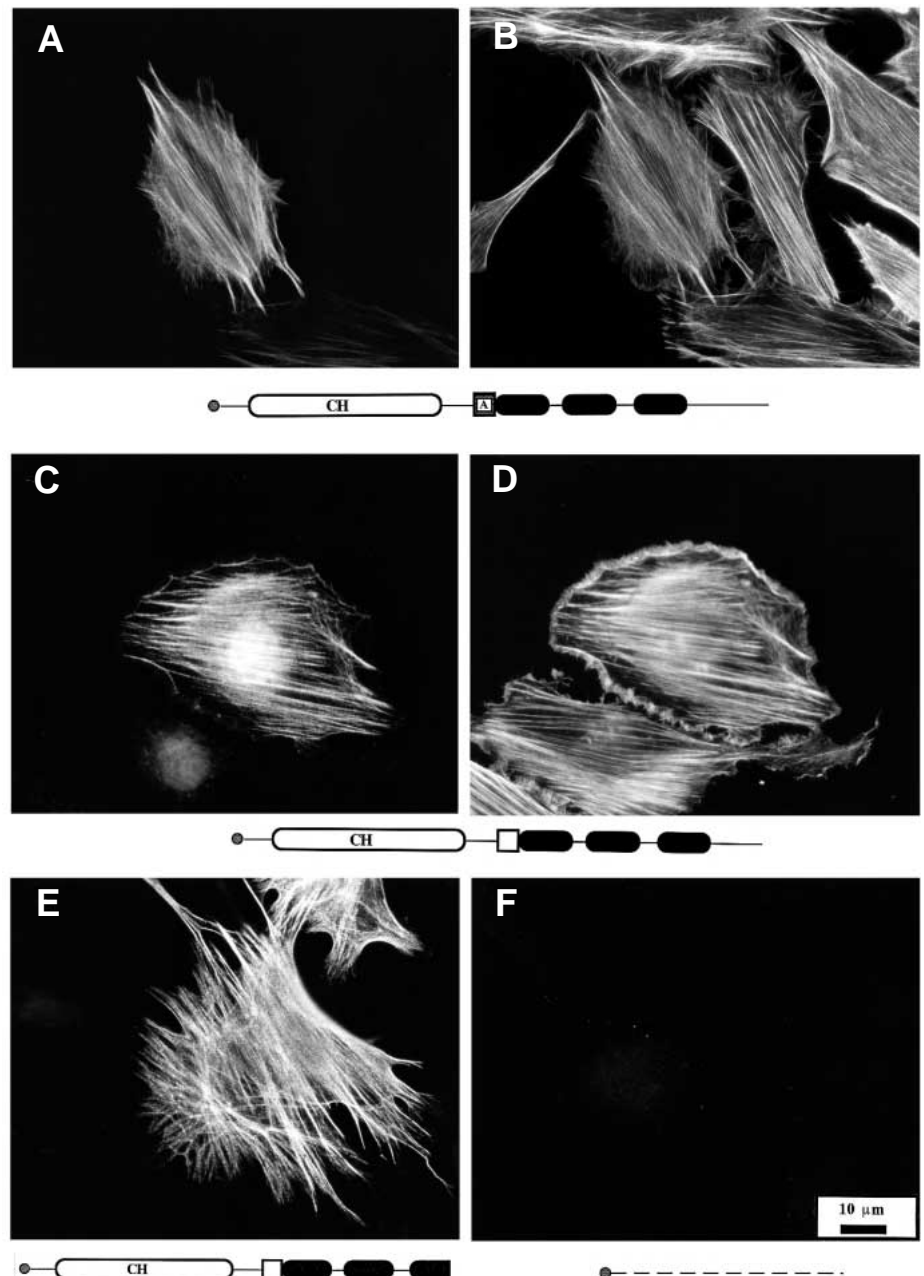


Fig. 6. Acidic and *h2* CaP colocalise with actin stress fibres in fibroblasts. Actin stress fibre localisation of full-length human acidic CaP (A,B), full-length mouse uterus *h2* CaP (C,D) and the carboxy-terminally truncated fragment *h2/1-262* (E). Deletion of the carboxy-terminal sequences in *h2* CaP increases stress fibre association for the latter construct. (F) HA-tag vector control; (A,C,E,F) anti-tag antibody/GaM Cy 3; (B,D) FITC-phalloidin. Schematic insets as in Fig. 5.

independent stable clones generated in NIH 3T3 cells. Furthermore, a carboxy-terminal truncation of SM 22 (SM 1-134) was found associated with vesicular structures (Fig. 7G,H).

Transient transfections of REF 52 cells with constructs including the amino terminus of *h1* CaP up to the end of the CH domain resulted in accumulation of the tagged protein fragment in endosomal and Golgi-like structures (Fig. 8A). Notably, the same result was obtained with a hybrid construct that contained two CH domains in tandem (Fig. 8B). By contrast, a construct composed of the three carboxyl terminal tandem repeats alone readily associated with actin stress fibers (Fig. 8C), an association that was as if this motif was duplicated (Fig. 8D), indicating that the calponin repeats form an autonomous actin binding domain.

DISCUSSION

To investigate the functional significance of two novel protein modules, namely the amino-terminal CH domain and the carboxy-terminal 29-residue tandem repeats of calponin, we have compared the contribution of these domains to actin binding. We have included SM 22 in this analysis since it contains a single CH domain and one calponin-like carboxy-terminal repeat (CLR), but lacks a consensus actin-binding motif as found in *h1* and acidic CaP. Thus, SM 22 was a suitable candidate for studying the targeting potential of the CH domain in a non-calponin molecule *in vitro*.

We have shown previously (North et al., 1994) that the smooth muscle variant of calponin (*h1*) associates with both contractile (γ -smooth) and cytoskeletal (β -cytoplasmic) actin

isoforms present in chicken gizzard muscle. Since β -cytoplasmic actin is the main actin isoform expressed in fibroblasts the observed binding of the *h1* and *h2* CaP constructs to the actin cytoskeleton in NIH 3T3 and REF 52 fibroblasts likely reflects an association with this non-muscle actin variant.

Although the sequence spanning residues 142-163 in *h1* CaP constitutes an active F-actin binding site and a peptide comprising this sequence can be cross-linked to and co-sediments with F-actin in vitro (Mezgueldi et al., 1992, 1995),

our findings show that the matching domain in *h2* CaP does not confer actin binding either in vitro or in cells in culture. Compared to the actin binding motifs in *h1* and acidic CaP, the domain in *h2* CaP differs in six and four positions, respectively, three of which appear to be significant as regards actin binding activity. One of these residues, Lys156 in *h1* CaP (Lys154 in the chicken α -CaP sequence), is critical for actin binding (Gong et al., 1993) and another, Ala145, for conferring the inhibitory activity of CaP on the actin-activated myosin ATPase (EL-Mezgueldi et al., 1996). Finally, the substitution

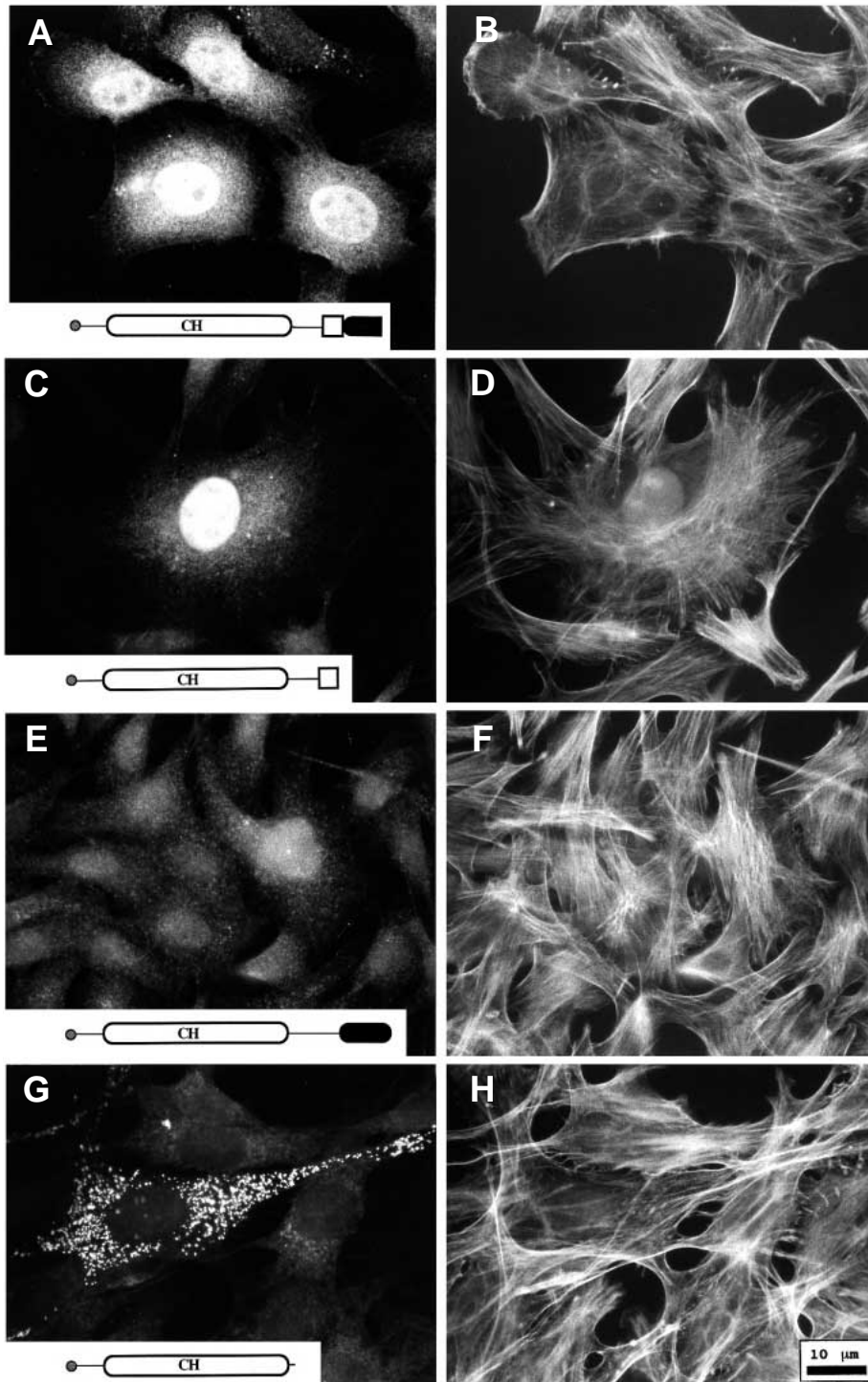
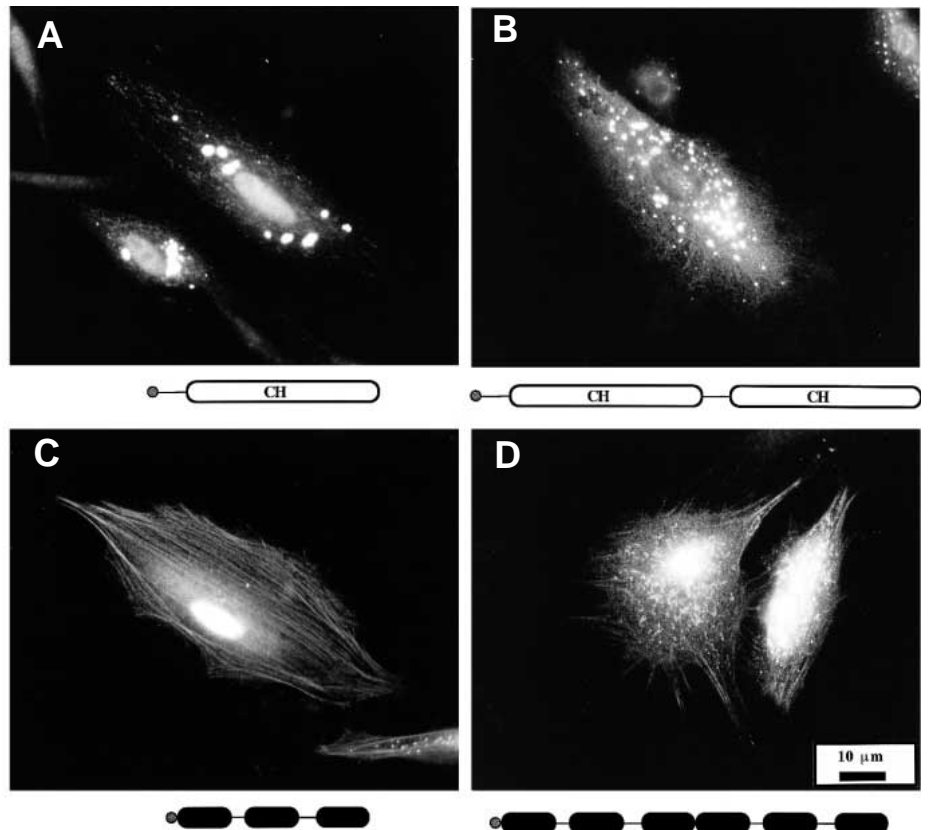


Fig. 7. The CH domain in *h2* CaP and SM 22 fails to mediate actin binding. Constructs *h2*/1-186 (A,B) lacking two, and *h2*/1-161 (C,D) lacking all three carboxy-terminal repeats, fail to colocalise with F-actin structures in NIH 3T3 cells transfected with the respective construct. Immunoreactive material is detected throughout the cytoplasm and also in the nucleus. A similar non-specific cytoplasmic distribution is found for full-length SM 22 (E,F). A carboxy-terminal truncation of SM 22 containing only the sequences corresponding to the CH domain in SM 22 and comprising residues 1-134, but missing the CaP-like repeat, is found accumulated in vesicular structures throughout the cytoplasm. (A,C,E,G) 12CA5/GaM Cy 3; (B,D,F,H) FITC-phalloidin. Schematic insets as in Fig. 5.

Fig. 8. The tandem repeats but not the single CH domains target to actin stress fibres. Immunofluorescence micrographs of REF 52 cells transiently transfected with constructs corresponding to the amino terminus (residues 1-127, A) or the tandem repeat domain (residues 164-1273, C) of *h1* CaP. Neither the single CH domain (A) nor a construct containing the *h1* CaP CH domain in tandem (B) associate with the actin cytoskeleton and immunoreactive material is concentrated in vesicular structures throughout the cell. By contrast the isolated tandem repeats (B) associate with the actin stress fibres, and this association is also retained in a duplication construct (D) containing six of the calponin repeats (see also Discussion). All anti-tag antibody/GaM Cy 3. Schematic insets as in Fig. 5.



of Arg151 to Asn removes a positive charge from this region in *h2* CaP. Our data are consistent with this region in *h2* calponin being inactive in actin binding.

While calponin and calponin-related proteins contain a single CH domain, two adjacent domains of this type are found in the actin binding domains of α -actinin, filamin, spectrin and fimbrin (Castresana and Saraste, 1995; Matsudaira, 1991; Pollard et al., 1994). From their X-ray crystal data, Carugo et al. (1997) proposed that both CH domains of spectrin contribute to F-actin binding and suggested that the juxtaposed amino- and carboxy-terminal helices of these two domains play a significant role in this interaction. The partial similarities in sequence and, more strikingly, in the predicted three-dimensional folding of the CH domains (McGough et al., 1994; Carugo et al., 1997; Goldsmith et al., 1997) with molecules involved in Rac and Cdc 42 signaling, like IQGAP 1 and 2, and Vav (Castresana and Saraste, 1995; Romero and Fischer, 1996), have now fueled speculations about a CH domain-mediated binding of these molecules to F-actin, thereby linking signal transduction events directly to the actin cytoskeleton (Hart et al., 1996; Brill et al., 1996). Consistent with this general idea, Bashour et al. (1997) recently demonstrated an association of IQGAP 1 with microfilaments in lamellipodia and membrane ruffles, but not with stress fibres of cultured cells, and Fukata et al. (1997) further confirmed the potential of IQGAP 1 to cross-link F-actin *in vitro*. Like calponin, however, these signalling molecules possess only one CH domain at their amino-terminal ends. Single CH domains likewise exist in the calponin-related proteins mp-20, np 25 and SM 22. Similar to the results obtained with constructs of *h1* and *h2* calponin containing only the CH domain, we were

unable to detect a direct association with F-actin for both full-length SM 22 (not shown) and the construct SM 22/1-134 in *in vitro* co-sedimentation and cell transfection assays. Furthermore, a construct containing two CH domains in tandem, mimicking an artificial actin binding-site, similar to that found in α -actinin and filamin, also failed to target stress fibres in transfected REF 52 fibroblasts.

Taken together, our results support the assumptions that (1) the presence of a single CH domain is not sufficient for the association with actin stress fibres of calponin *in vivo* and/or (2) the significant divergence in sequence between the individual CH domains of the different CH domain-containing proteins reflect functional differences of this protein module (T. Stradal, W. Kranewitter, S. J. Winder and M. Gimona, manuscript in preparation). It is, however, conceivable that the region embracing the CH domain in CaP and other single CH domain-containing proteins cooperates with a second site in thin filament-binding. This is also suggested by density maps of reconstituted thin filaments (Hodgkinson et al., 1997), which show that the CH domain of CaP occupies a position along the actin filament identical to that observed for spectrin (Schutt et al., 1997) and fimbrin (Hanein et al., 1997). In light of our data one may therefore speculate that F-actin association via a CH domain requires cooperativity with either a second CH domain, a tandem-repeat domain, or other domains containing a polycationic stretch like those found in PH domains (see Musacchio et al., 1993). Thus, CH domains may define a novel family of weak actin-binding modules, which act synergistically with other modules to mediate F-actin binding.

Although constructs of *h1* and *h2* CaP containing only the

amino-terminal part of the calponin molecule, embracing the CH domain, failed to associate with F-actin, those that included the carboxy-terminal tandem-repeat sequences retained their actin-binding ability. Moreover, the interaction of the isolated calponin tandem-repeat region spanning residues 164-273 of *h1* CaP with actin filaments identifies this region as a hitherto unrecognised actin-binding domain in *h1* CaP. This conclusion is further supported by our findings that proteins like SM 22 associate with F-actin in transfected fibroblasts when fused to the calponin tandem-repeat motif (R. Mital and M. Gimona, manuscript in preparation). Notably, the product of the *unc-87* gene from *C. elegans* has no recognisable actin-binding domain, but contains seven copies of the calponin-like repeats, suggesting that these CLR's are responsible for the observed association of the UNC-87 protein with sarcomeric actin-containing structures (Goetinck and Waterston, 1994b).

While this manuscript was under revision, Mino and colleagues (Mino et al., 1998) demonstrated that a synthetic peptide corresponding to residues 172-187 of the *h1* CaP sequence binds to F-actin *in vitro* but does not inhibit the actin-activated ATPase activity of myosin, and the authors propose, in agreement with our data, a second independent actin binding site in smooth muscle calponin proximal to the actin binding motif identified earlier.

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